The phage abortive infection system, ToxIN, functions as a protein–RNA toxin–antitoxin pair

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Various mechanisms exist that enable bacteria to resist bacteriophage infection. Resistance strategies include the abortive infection (Abi) systems, which promote cell death and limit phage replication within a bacterial population. A highly effective 2-gene Abi system from the phytopathogen *Erwinia carotovora* subspecies *atroseptica*, designated ToxIN, is described. The ToxIN Abi system also functions as a toxin–antitoxin (TA) pair, with ToxN inhibiting bacterial growth and the tandemly repeated ToxI RNA antitoxin counteracting the toxicity. TA modules are currently divided into 2 classes, protein and RNA antisense. We provide evidence that ToxIN defines an entirely new TA class that functions via a novel protein-RNA mechanism, with analogous systems present in diverse bacteria. Despite the debated role of TA systems, we demonstrate that ToxIN provides viral resistance in a range of bacterial genera against multiple phages. This is the first demonstration of a novel mechanistic class of TA systems and of an Abi system functioning in different bacterial genera, both with implications for the dynamics of phage-bacterial interactions.

Bacteriophage | Bacteriostasis | Erwinia | plasmid | resistance

Bacteria, the most abundant organisms on the planet, constantly face challenges from their own viral parasites, bacteriophages. Outnumbered approximately 10 to 1 by the estimated $\geq 10^{30}$ phages on Earth (1, 2), bacteria become infected at rates of 10^{25} per second (3). The rapid turnover of such large quantities of organic material impacts on nutrient cycling and the global climate (4, 5). This global predator–prey relationship is an evolutionary clash that has forced bacteria to develop multiple methods of protection (6). These include surface alterations to avoid phage adsorption, prevention of phage DNA injection, restriction of incoming DNA, acquiring phage-specific immunity through clustered regularly interspaced short palindromic repeats (7) and abortive infection (Abi). Abi systems provide population protection by promoting "altruistic suicide" of an infected bacterium (8). The majority of Abis have been found on plasmids of Gram-positive lactococcal strains (9), but some have been found in Gram-negative species, including *Escherichia coli*, *Vibrio cholerae*, and *Shigella dysenteriae* (10–12). Abi systems often are highly toxic when activated; they have varied targets and can act on central cellular processes to inhibit phage DNA replication, transcription, and protein synthesis (9). Specific effects include premature cell lysis by AbiZ (13) and interference of a phage RuvC-like endonuclease by AbiD1 (14).

Toxic proteins play many roles within bacteria. The recent influx of genomic information has allowed frequent identification of multiple "toxin–antitoxin" (TA) loci on the chromosomes of both bacteria and archaea (15). Although originally identified as plasmid addiction systems (16), the apparent widespread nature of these TA operons has led to discussion of the biological role of such systems (17). TA systems rely on the dual activity of a toxin and an antagonistic antitoxin (18). Antitoxins are labile compared with their toxins, and when production of both components is inhibited, the antitoxin is turned over preferentially, allowing the toxin to take effect (18). The toxins of known TA systems, similar to Abi proteins, can target central cellular processes such as DNA replication and translation by inhibiting DNA gyrase (*ccd* and *parDE* loci) and causing mRNA degradation (*relBE* and *mazEF* loci), respectively (18). TA loci are thought to fall into 2 categories, protein–protein systems, such as Phd-Doc (18), and RNA–RNA systems, such as *hok* (host killing)/*sok* (suppressor of killing) (19).

Here we identify a cryptic plasmid of the Gram-negative phytopathogen *Erwinia carotovora* subspecies *atroseptica* (*Eca*) 1039, carrying a gene encoding a protein with sequence identity to AbiQ of *Lactococcus lactis* (20). This *Eca* homologue, designated ToxN (for toxin), protects against multiple phages through abortive infection. Controlled expression of ToxN is bacteriostatic, and toxicity of the ToxN protein is suppressed by the product of an upstream gene, *toxI* (ToxN inhibitor), encoding an antitoxic RNA. Therefore, together, ToxIN acts as a novel protein–RNA TA pair, the first described TA system of this type. We further show that there are widespread homologues of this new class of TA system in diverse phyla, and that the antiphage activity is maintained within multiple enteric genera.

Results

An *Eca* Plasmid Provides Phage Resistance. *Eca* causes soft-rot and blackleg disease of potatoes (21). A 5620-bp cryptic plasmid, pECA1039, was isolated from *Eca* 1039 and sequenced. Bioinformatic searches identified a ColE1-type replication origin and up to 11 predicted ORFs (supporting information (SI) Table S1 and Fig. 1*A*). The product of the third predicted ORF, designated ToxN, has 31% identity to AbiQ, an Abi protein from *L. lactis* W-37 plasmid pSRQ900 (20). The *toxN* gene was 3′ of a gene annotated de novo (designated *toxI*) and predicted to be operonic with *toxN*.

As we have no phages able to infect *Eca* 1039, 3 pECA1039 subclones (pECA1039–1, -2, and -3) were used to transform *Eca* strain 1043, and the transformants were tested for phage resistance against phages ϕA2 and ϕM1 (22). Plasmid pECA1039–1 provided protection from ϕA2 and ϕM1 (Fig. 1*B*), but pECA1039–2 and pECA1039–3 did not (data not shown). Furthermore, a *toxN* frameshift (FS) mutation in pECA1039–1 abolished the phage-resistance phenotype (Fig. 1*B*). To determine whether the native replicon provided phage resistance, in vitro transposon mutants of pECA1039 were generated. In *Eca* 1043, plasmid pECA1039-Km12 provided resistance to ϕA2

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Data Deposition: The sequence reported in this paper has been deposited in the GenBank database (Plasmid pECA1039, accession no. FJ176937).

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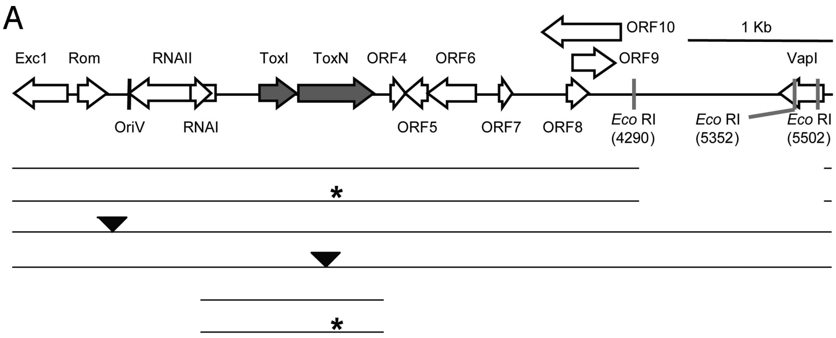

B

Plasmid	EOP ΦA2	EOP ΦM1
pECA1039-1	7.5 x 10^{-8}	7.9 x 10^{-6}
pECA1039-1-FS	0.8	0.6
pECA1039-Km12	4.2 x 10^{-5}	0.6
pECA1039-Km23	1.5	0.8
pTA46	6.6 x 10^{-6}	1.3 x 10^{-5}
pTA47	1.5	0.9

Fig. 1. *toxIN* on plasmid pECA1039 encodes a phage-resistance system. (*A*) Linear map of pECA1039 and subsequent constructs, together with (*B*) EOP data of each construct versus ϕA2 and ϕM1. Here * denotes a frameshift mutation, and ▼ denotes a transposon insertion site.

(Fig. 1*B*), but only a reproducible reduction in plaque size for ϕM1 (data not shown). Why pECA1039-Km12 affects the size but not number of ϕM1 plaques is unclear. Plasmid pECA1039-Km23 has a transposon insertion within *toxN*, which abolished phage resistance (Fig. 1*B*). A smaller subcloned region consisting of *toxN* and *toxI* provided protection from phage infection, and this response was removed by a FS mutation within *toxN* (pTA46 and pTA47, respectively; Fig. 1*B*). Therefore, the *toxIN* locus on pECA1039 encodes an effective phage-resistance system.

***toxIN* Encodes an Abi System.** The protein sequence identity between ToxN and AbiQ from *L. lactis* suggests that the *toxIN* locus might encode a phage Abi system, and this hypothesis was tested (Table S2). Adsorption of phages ϕA2 and ϕM1 to *Eca* 1043 was unaffected by the presence of a *toxIN* plasmid, compared with *toxI*, *toxN*-FS, and vector-only control plasmids. Moreover, ϕA2 and ϕM1 "escape" mutant phages were isolated at a low frequency and were nonresponsive to *toxIN*. Therefore, to evaluate whether *toxIN* encoded a restriction-modification system, phages that were able to overcome the resistance system were passaged twice through *Eca* 1043 before retitrating on *Eca* 1043 containing *toxIN*. These phages retained their insensitivity to *toxIN*, indicating a stable genetic resistance mechanism and not phenotypic escape from a restriction-modification system (data not shown). The survival of *Eca* 1043 infected with ϕA2 and ϕM1 was unaffected by the presence of *toxIN*. Finally, *toxIN* dramatically reduced the burst size and efficiency of center of infection (ECOI) formation of ϕA2 and ϕM1 on *Eca* 1043. All of our data are consistent with ToxIN functioning as a phage Abi system (Table S2).

ToxN Is a Toxin in *E. coli*. Initial attempts to clone *toxN* under nonnative promoters yielded only mutant *toxN* clones, suggesting that the gene product was toxic in *E. coli*. Indeed, induction of *toxN* expression using the P_{araBAD} promoter in *E. coli* resulted in growth cessation, as measured by OD_{600} (Fig. 2*A*), along with a $\approx 1 \times 10^6$ reduction in colony- forming units (cfu) per mL (Fig. 2*B* and *C*). Induction of a *toxN*-FS strain had no effect, demonstrating that the ToxN protein was required for growth inhibition. Furthermore, *E. coli* expressing ToxN displayed no obvious morphological differences compared with the *toxN*-FS control under combined bright field and fluorescence microscopy (data not shown). Therefore, expression of the ToxN protein was growth-inhibiting to *E. coli* and did not result in cell lysis.

***toxIN* Is Bicistronic.** The *toxI* gene is composed of 5.5 almost-identical direct repeats of 36 nucleotides, followed by a predicted rho-independent transcriptional terminator (Fig. 3*A*). Within this region is a predicted ORF with a rare TTG start codon but no similarity to any other predicted protein. The transcriptional start site (+1) of *toxI* was mapped and found to be preceded by putative -10 and -35 promoter elements related to the *E. coli* σ^{70} consensus (Fig. 3*A*). In a low-copy *lacZ* promoter probe vector,

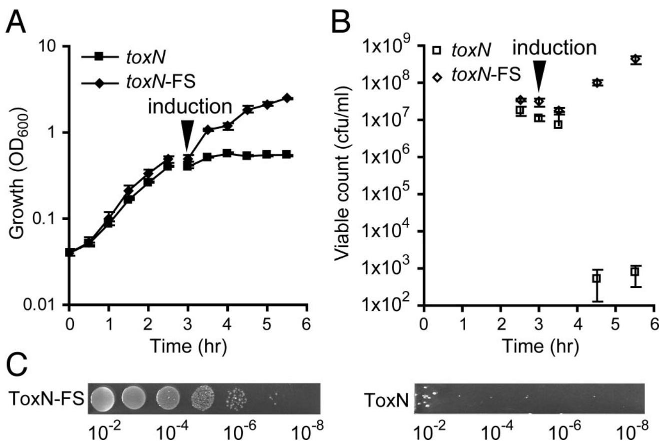

Fig. 2. ToxN is growth-inhibiting. (*A*) Growth (OD_{600}) and (*B*) viable counts of *E. coli* DH5α were measured after induction of the *toxN* gene (pTA49) or a *toxN*-FS control (pTA50); for details, see *Materials and Methods.* (*C*) Serial dilutions of exponentially grown cultures of *E. coli* DH5α with ToxN-FS (pTA50) or ToxN (pTA49) plated on LBA, Ap, and L-ara (inducing conditions).

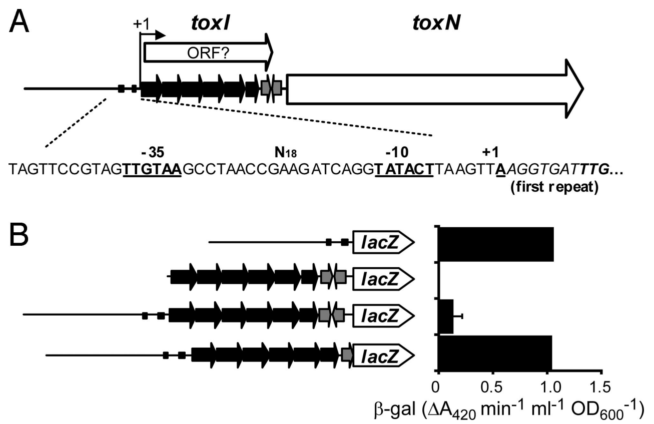

Fig. 3. Organization of the *toxIN* locus. (*A*) Schematic representation of the *toxIN* locus. The transcription start (+1), *toxI* tandem repeats (black arrows), rho-independent terminator (gray arrows), *toxN* gene (white arrow), and promoter -35 and -10 elements are indicated. The hypothetical *toxI* ORF also is shown. (*B*) *toxIN* promoter *lacZ* transcriptional fusions in *E. coli* DH5α using plasmids (from top to bottom) pTA104, pTA105, pTA106, and pTA119.

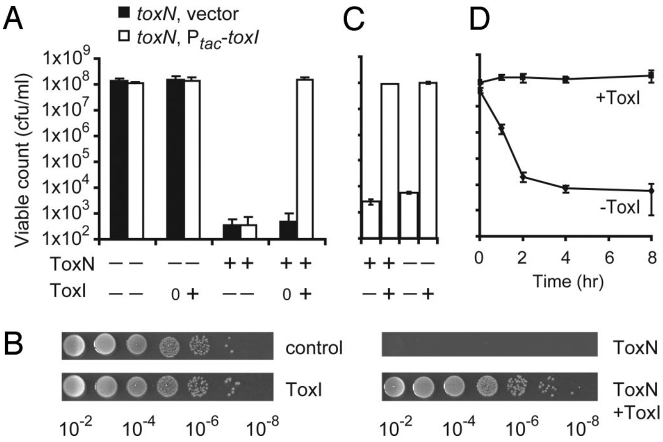

Fig. 4. The *toxIN* locus encodes a bacteriostatic TA system. (*A*) Protection of *E. coli* DH5α from ToxN inhibition by transcription of *toxI*. Protection assays were conducted as described in *Materials and Methods*, and the strains shown are *E. coli* DH5α, pTA49, pTA100 (*toxN*, vector) and *E. coli* DH5α, pTA49, pTA76 (*toxN*, P_{tac}-*toxI*). The symbols "+" and "−" refer to induction or repression of *toxN* (L-ara and glu) and *toxI* (+/− IPTG). Empty vector induction is indicated by 0. (*B*) Serial dilutions of *E. coli* DH5α, pTA49, pTA76 on LBA plates with Ap, Sp and, glu (control), glu and IPTG (ToxN), L-ara (ToxN), and L-ara and IPTG (ToxN+ToxI). (*C*) ToxI is less stable than ToxN. *E. coli* DH5α, pTA49, pTA76 was grown expressing both ToxI and ToxN, as described in *Materials and Methods*, and plated under conditions resulting in continued expression or repression of either ToxI or ToxN or both (+ and −). (*D*) ToxN is bacteriostatic. ToxN was induced in *E. coli* DH5α, pTA49, pTA76 for different times, and viable counts were determined on LBA Ap, Sp, and glu plates without (ToxN) or with (ToxN+ToxI) IPTG. Time (hr) refers to hours after ToxN induction.

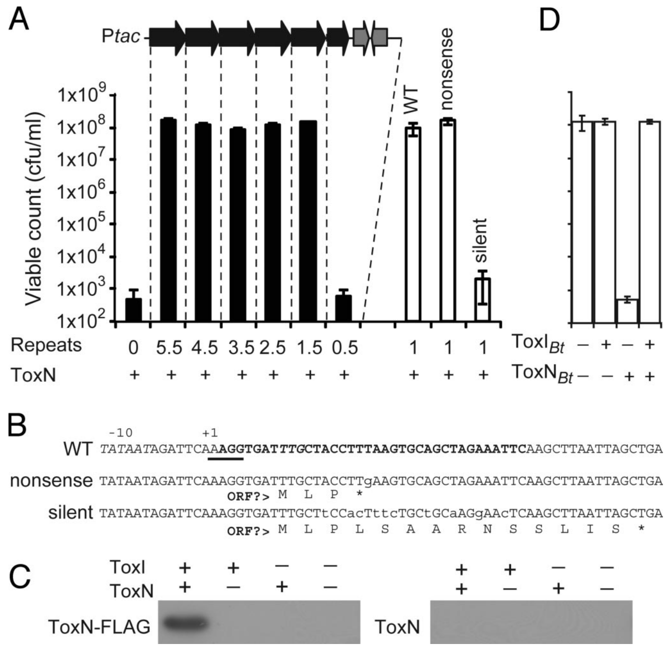

Fig. 5. ToxIN is an RNA-protein TA system. (*A*) Protection of *E. coli* DH5α from ToxN inhibition (pTA49) by expression of *toxI* deletions composed of 5.5 (pTA76), 4.5 (pTA78), 3.5 (pTA79), 2.5 (pTA80), 1.5 (pTA81), 0.5 (pTA93), a WT single 36-nt repeat (pTA103), a nonsense 36-nt repeat mutant (pTA122), or a silent 36-nt repeat mutant (pTA107). (*B*) Sequences of the 36-nt inserts in pTA103 (WT), pTA122 (nonsense), and pTA107 (silent) and the putative short peptides that they may encode. A putative ribosome binding site is underscored, the possible start codon is in italic type, and the single repeat is in bold type. (*C*) Expression of the ToxI RNA results in the stable production of ToxN. *E. coli* DH5α, pTA76, pTRB1 and *E. coli* DH5α, pTA76, pTA49 were grown, and samples were probed with a polyclonal anti-FLAG antibody as described in *Materials and Methods*. (*D*) Protection of *E. coli* DH5α from $ToxN_{Bt}$ inhibition by transcription of $toxI_{Bt}$. Protection assays were performed as described in *Materials and Methods*; the strain shown here is *E. coli* DH5α, pTA117, pTA115 ($toxN_{Bt}$, P_{tac}-$toxI_{Bt}$). The symbols "+" and "−" refer to induction or repression of *toxN* (L-ara and glu) and *toxI* (+/− IPTG).

the promoter had moderate expression in *E. coli* (Fig. 3*B*). Further experiments indicated no detectable *toxN* promoter within *toxI*. Transcriptional read-through into *toxN* from the *toxI* promoter past the terminator was detected, however (Fig. 3*B*). The presence of a read-through transcript from the mapped +1 into *toxN* was confirmed by RT-PCR (data not shown); thus, these 2 genes are cotranscribed, with the majority (≈90%) of transcriptions terminating at the rho-independent hairpin.

***toxI* Encodes an Antitoxin.** The *toxIN* genetic organization and toxic nature of *toxN* suggested that this locus might encode a TA system with *toxI* providing the antitoxin function. When cloned under an inducible promoter (P_{tac}), induction of *toxI* expression provided antitoxin activity, demonstrating that *toxI* transcription was necessary for the repression of ToxN toxicity (Fig. 4*A* and *B*). The toxicity of ToxN in *Eca* 1043 also was prevented by *toxI* expression, and co-overexpression of ToxI and ToxN from separate plasmids was found to confer phage resistance (data not shown). Next, to examine whether the antitoxin, ToxI, was less stable than ToxN, both components were expressed, and then either component or both components were switched off. As expected, when ToxN was either continuously expressed or turned off in the presence of ToxI, no decrease in viable count was observed (Fig. 4*C*); however, when both components were switched off, the viable count decreased by $>1 \times 10^4$ (Fig. 4*C*), suggesting that ToxI is less stable than ToxN. In summary, these results demonstrate that the *toxIN* operon encodes a TA system.

ToxIN Is a Reversible Bacteriostatic TA System. The lack of ToxN-induced bacterial lysis prompted an investigation into whether or not ToxN is bacteriostatic. Delayed overexpression of *toxI* enabled full recovery of *E. coli* cells that had been expressing ToxN for at least 8 h (Fig. 4*D*). Furthermore, cells that had transiently expressed ToxN did not grow on plates within 1 day, due to the presence of the toxin. But after 2 days of incubation, colonies arose, presumably due to turnover of ToxN (data not shown). This finding indicates that ToxN functions through a reversible growth-inhibiting (bacteriostatic) mechanism.

ToxIN Is a Novel Protein–RNA TA System. TA systems are broadly classified into protein–protein and RNA antisense groups. Whether or not *toxI* encoded a protein was examined. First, *toxI* was tagged with C-terminal hexahistidine sequences. These constructs were functional in protection assays, but no ToxI protein was detectable by Western blot analysis (data not shown). Next, plasmids were constructed that enabled the expression of RNA species with 5.5, 4.5, 3.5, 2.5, 1.5, and 0.5 repeats, followed by the native rho-independent transcriptional terminator. All plasmids with at least 1.5 "repeats" could protect *E. coli* from ToxN (Fig. 5*A*). In addition, expression of only a single 36-nucleotide (nt) repeat could inhibit ToxN toxicity (Fig. 5*A* and *B*). It was possible that the single 36-nt RNA was encoding a small peptide that inhibits ToxN. To test this, a single nt nonsense mutation was generated in this 36-nt sequence that would terminate translation at the putative fourth codon. This plasmid could still protect *E. coli* from ToxN, whereas a plasmid with multiple silent point mutations in the ToxI RNA, which in theory could still code for the same hypothetical peptide, was nonfunctional (Fig. 5*A* and *B*). Therefore, *toxI* encodes an RNA antitoxin, with the minimum functional unit currently defined as a single 36-nt repeat.

RNA antitoxins function through an antisense base-pairing mechanism that results in inhibition of toxin translation. To enable analysis of ToxN protein levels, we extended the *toxN* ORF to encode a C-terminal FLAG epitope. We then performed toxicity and phage-resistance assays, which confirmed that this C-terminal FLAG epitope does not affect ToxN toxicity or reduce phage resistance conferred by ToxN (data not shown). Surprisingly, coexpression of ToxI and ToxN resulted in detectable levels of ToxN protein when assessed by SDS/PAGE (data not shown) and Western blot analysis (Fig. 5*C*). The identity of the ToxN band separated by SDS/PAGE was verified by MS analysis (70% coverage). Overexpression of *toxN* alone did not lead to detectable levels of the FLAG-tagged ToxN protein (Fig. 5*C*). Therefore, *toxI* encodes an RNA (ToxI) that inhibits the toxic effects of the ToxN protein. To the best of our knowledge, this is the first reported example of a protein–RNA TA system.

ToxIN Defines a New TA Family. The novel mechanism of ToxIN prompted us to examine the phylogenetic distribution of this TA module, which resulted in the identification of at least 19 ToxN homologues, including ToxN itself (Table S3). Genes predicted to encode ToxN-like proteins were found in Gram-positive and -negative bacteria across a range of bacterial phyla. ToxN has no sequence similarity to characterized protein domains, and thus it is the defining member of this new toxin class. Of the 18 *toxN*-like genes with 5′ sequences available, 16 had putative RNA antitoxin species with near-consensus promoters and predicted rho-independent terminators, and 13 of these had distinctive repeats (Table S3). The antitoxin sequences are not conserved in sequence or predicted structure (data not shown), although some closely related *toxN*-like genes are associated with near-identical repeats (e.g., *Haemophilus influenzae* plasmids) (Table S3). The TA gene organization is conserved in all detected members of this proposed novel class of protein–RNA TA systems.

To test whether the *toxN*-like genes and their predicted antitoxin RNAs also functioned as protein-RNA TA systems, we examined *toxIN* from *Bacillus thuringiensis* serovar *kurstaki* strain HD73, plasmid pAW63 ($toxIN_{Bt}$). Expression of $ToxN_{Bt}$ in *E. coli* caused a dramatic reduction in viable count that could be inhibited by expression of the antitoxin RNA, $ToxI_{Bt}$ (Fig. 5*D*), which has 3.1 near-identical repetitive sequences of 34 bp (Table S3).

***toxIN* Acts as an Abi System in Multiple Enteric Bacteria.** The distribution of *toxIN*-like loci motivated us to test the host range of ToxIN activity. First, we demonstrated that ToxIN could provide resistance in *Eca* 1043 against a suite of phages. Of 25 *Eca* phages (including ϕA2 and ϕM1), 13 were sensitive [here defined as an efficiency of plaquing (EOP) $\leq 1 \times 10^{-2}$] to ToxIN (pTA46), with the full-suite EOP ranging from 1 to $\leq 1 \times 10^{-10}$. Next, we found that in *E. coli* DH5α, none of the 4 coliphages tested (Mu, λ*vir*, P1*vir*, and T4) was affected by *toxIN* (pTA46), but 5 of 10 newly isolated coliphages (ϕTB16–25) were sensitive to ToxIN, with EOPs $\leq 1 \times 10^{-8}$. Finally, in *Serratia marcescens* Db11, of 11 phages tested, 3 were sensitive to *toxIN* (pECA1039Km12 and Km15; reduced plaque size, EOP $= 0.5$ to $< 1 \times 10^{-7}$). This clearly indicates that *toxIN* encodes a TA/Abi system active against a range of phages within multiple enteric genera.

Discussion

We have identified and characterized the *Eca* plasmid-encoded *toxIN* locus, which directs abortive infection of multiple phages in various enteric bacteria. Our data demonstrate that *toxIN* functions as an Abi system (Fig. 1 and Table S2). Furthermore, we have shown that *toxIN* defines a new family of novel TA systems proposed to act through an RNA–protein interaction, an entirely new mechanistic class of TA systems separate from the well-characterized proteic (15, 18) and RNA antisense groups (19).

The toxic effects of ToxN were inhibited by expression of a gene transcribed immediately 5′ of *toxN*, designated *toxI*. This genetic organization is common to many TA modules. Together, the ToxI and ToxN products constitute a TA system with an antitoxin product, ToxI, apparently of lower stability than ToxN (Fig. 4). The *toxIN* genes are bicistronic, with most expression terminating after *toxI*, leaving ≈10% of read-through into *toxN*. Termination might be important for obtaining an appropriate ToxI:ToxN stoichiometry. Surprisingly, the growth-inhibiting effect of ToxN was bacteriostatic (Fig. 4), and ToxI could resuscitate *E. coli* in the absence of new ToxN synthesis, suggesting that ToxI may be able to compete efficiently with the cellular target(s) of ToxN. Our results are similar to those observed for RelE and MazF toxins expressed for up to 4 or 5 h, respectively (23); however, another study demonstrated a "point of no return" after overexpression of MazF, whereby MazE could rescue most bacterial growth only within 6 h (24).

The *toxI* gene is composed of 5.5 directly repeated sequences and contains a small putative ORF. But evidence supports the assignment of an RNA species as the active antitoxin component: (*i*) transcription of only a single repeat RNA of 36 nt retained full activity (Fig. 5); (*ii*) a nonsense point mutant variant that terminated the putative ORF remained functional, whereas a silent mutant variant that had unaltered peptide coding was inactive (Fig. 5); (*iii*) the predicted transcribed regions 5′ of most other *toxN* genes contain no predicted ORFs (data not shown); (*iv*) $ToxI_{Bt}$ from *Bacillus thuringiensis* was an active antitoxin against $ToxN_{Bt}$ but had no putative coding region (Fig. 5); and, finally, (*v*) overexpression of hexahistidine-tagged ToxI protected against ToxN with no detectable ToxI protein production.

The RNA nature of ToxI suggests that the *toxIN* system is an RNA antisense TA module; however, when ToxI RNA was overexpressed, the ToxN protein was detectable (Fig. 5). Clearly, the *toxIN* system cannot be described by RNA antisense or proteic TA systems and thus requires a new classification. We propose a new class of TA modules requiring an RNA antitoxin predicted to interfere with the biochemical activity of the protein toxin. Our bioinformatic analyses identified numerous ToxIN-type loci in diverse bacterial phyla and supported an organizational and functional link between the predicted RNA antitoxins and the ToxN-like genes. For example, 2 *toxN* pseudogenes identified in *Histophilus somni* strain 129PT displayed antitoxin degeneration (data not shown) and another putative ToxIN system, that from *B. thuringiensis* plasmid pAW63, functioned as a TA module in *E. coli* (Fig. 5).

Most *toxIN*-like genes are plasmid-encoded, and their phylogenetic distribution implies a role of horizontal gene transfer in their dissemination. Indeed, the *B. thuringiensis* plasmid pAW63 is conjugal (25). We were interested in mimicking the acquisition of the *toxIN* system by different bacterial genera and the effects on phage resistance. Our study provides the first evidence that an Abi (or TA) system can impart protection in different genera against multiple phages. This has implications for the dissemination and action of Abi/TA systems, and the technical utility of such a broad-spectrum system (with respect to both host and phages) has not escaped our attention.

Despite extensive recent research, debate continues about the biological roles of TA systems (reviewed in ref. 17). One suggested function of TA systems is to reduce phage infection; in fact, the *hok*/*sok* loci from *E. coli* plasmid R1 excludes phage T4 (26), and the chromosomal TA system, *mazEF*, protects against phage P1 (27). The *toxIN* genes abort the infections of different phages and function in a number of different genera; therefore, chromosomal or plasmid-encoded representatives across 3 TA classes [proteic (*mazEF*), RNA antisense (*hok*/*sok*), and protein–RNA (*toxIN*)] can function to limit phage infection.

MICROBIOLOGY

This study demonstrates that the effect of TA modules as antiphage elements may be an evolutionarily important, widespread phenomenon. This is particularly important in light of the huge numbers of phages in the environment (2) and the strong selective pressure on bacteria to develop phage-resistance mechanisms (28). But TA elements can have biological roles in the absence of phages (e.g., plasmid stabilization) (17), and phages may provide further selective pressure for the maintenance of these genes in some circumstances. Interestingly, *toxIN* also can provide plasmid stabilization (P.F., unpublished data).

To the best of our knowledge, this is the first case of an Abi system that functions as a TA module, blurring the boundary between these systems. Some features of other Abi systems show similarities to TA modules. Some Abi systems require 2 protein components [e.g., AbiE (29), AbiG (30), AbiL (31), and AbiT (32)], and others may have RNA antitoxins presumably overlooked by standard gene sequence analysis [e.g., *abiQ* (20)]. In addition, numerous Abi proteins are toxic when expressed in the absence of phages [e.g., AbiD1 (33), AbiB (9), and AbiK (34)]. Analogously, restriction-modification systems, thought to function primarily in phage defense, also can function as TA modules for plasmid stabilization (35).

The antiphage activity of the ToxN homologue AbiQ is characterized by a late-acting step that prevents the processing of accumulated phage DNA (20). ToxN also prevents mature phage particle formation after normal phage DNA accumulation (Table S2 and T.B., unpublished data). Based on our current understanding, we propose an extended model for ToxIN antiphage activity. Before phage infection, transcription of the *toxIN* locus results in an excess of the unstable ToxI RNA relative to the ToxN protein. The ToxI RNA is predicted to interact directly with ToxN and inhibit toxicity. On phage infection, alterations in host transcription or translation or the degradation of bacterial DNA could destabilize the ToxI:ToxN ratio, freeing ToxN to interact with its target(s) to inhibit growth. Alternatively, a specific phage product could interact directly with ToxIN and trigger this system; for example, a phage antitermination mechanism may act to increase ToxN levels. We favor the first hypothesis because of the action of *toxIN* against multiple phages in different hosts, the observed sequence diversity in the phage gene pool (3), and the observation of no change in ToxN levels after phage infection (T.B., unpublished data). Due to its putative ToxI RNA binding, ToxN might function in the absence of ToxI to target cellular RNA [e.g., MazF from *E. coli* (36)]. It remains unclear how this can lead to the prevention of phage DNA processing. Research into *toxIN* will provide novel information about the mechanistic features of this new class of TA systems and insight into phage–host interactions.

Materials and Methods

Bacterial Strains, Plasmids, and Culture Conditions. *Eca* 1043 (21) and 1039 (37) were grown at 25°C, *E. coli* DH5α (Gibco/BRL) was grown at 37°C, and *S. marcescens* Db11 (38) was grown at 30°C in Luria broth (LB) at 300 rpm or on LB agar (LBA) containing 1.5% (w v^{-1}) agar, and growth (OD_{600}) was measured as described previously (39). When required, LB was supplemented with the following antibiotics: kanamycin 50 μg mL^{-1}, spectinomycin (Sp) 50 μg mL^{-1}, ampicillin (Ap) 100 μg mL^{-1}, and tetracycline 35 μg mL^{-1}. When required, D-glucose (glu) at 0.2% (w v^{-1}), L-arabinose (L-ara) at 0.1% (w v^{-1}), and isopropyl-β-D-thiogalactopyranoside (IPTG) at 1 mM were used, unless stated otherwise. All experiments were performed at least in triplicate (unless stated otherwise) and plotted as mean $\pm$ SD.

DNA Manipulations and Sequence Analysis. Molecular biology techniques and sequencing were performed as described previously (39). Named primers are listed in Table S4. All plasmids were verified by DNA sequencing. Sequence data were analyzed using GCG (Genetics Computer Group, University of Wisconsin), and ToxN homologues were identified using BLAST and PSI-BLAST. Direct repeats were identified using Tandem Repeats Finder (40), and transcriptional terminators were detected using STEMLOOP in GCG. ORFs were predicted by Genemark.hmm (41) and ORF Finder (http://www.ncbi.nlm.nih.gov/projects/gorf/).

Subcloning and Sequencing of pECA1039. Plasmid pECA1039 was extracted from *Eca* 1039 and cloned into EcoRI-digested pUC19. Three pECA1039 EcoRI subclones in pUC19 (pECA1039–1, -2, and -3) were sequenced. pECA1039 was completed by sequencing across the EcoRI junctions. pECA1039–1 was digested with BsmI, the 3′ overhang was removed with T4 polymerase, and ligated. The resulting plasmid (pECA1039–1-FS) has a 2-bp deletion, causing a premature stop codon after L114 in the ToxN protein. The *toxIN* genes and *toxI*, *toxN*-FS controls were cloned by PCR into pBR322 EcoRI and HindIII sites with primers MJ7 and KD02 using pECA1039–1 and pECA1039–1-FS, respectively, as template DNA, producing pTA46 (*toxIN*) and pTA47 (*toxI*, *toxN*-FS).

In Vitro Mutagenesis of pECA1039. In vitro transposon mutagenesis was performed on plasmid pECA1039 with EZ::TN™ <NotI/KAN-3>, following the manufacturer's instructions (Epicentre). Insertion sites of the EZ::TN™ <NotI/KAN-3> transposons were mapped by sequencing using primer PF134.

Phage Techniques. Phage resistance (i.e., EOP) was calculated after overnight incubation of phages in a 0.35% agar lawn of bacterial host using (phage titer on test host/phage titer on control host). Adsorption assays were performed as follows. A 10-mL bacterial culture was adjusted to OD_{600} = 1, inoculated with phages at a multiplicity of infection of 0.01, and incubated at 25°C at 300 rpm. Samples were obtained at 0 min and 30 min (ϕA2) or at 40 min (ϕM1), and each sample was added to 900 μL of phage buffer. After centrifugation for 10 min at 16,200 $\times$ *g*, 10 μL of the supernatant was taken for titer determination. Percentage adsorption was calculated as the percent change in supernatant titer from 0 to 30/40 min. Cell survival, ECOI, and burst size assays were performed as described previously (20).

ToxN Toxicity Assays. The *toxN* and *toxN*-FS genes were cloned by PCR into pBAD30 (42) EcoRI and HindIII sites using primers PF137 and KD02, with pECA1039–1 and pECA1039–1-FS, respectively, as template DNA. Transformants of all pBAD30 clones were selected on LBA, Ap, and glu to repress expression of the P_{araBAD} promoter. The resulting constructs enabled controlled expression of native, untagged ToxN (pTA49) and ToxN-FS (pTA50).

Cultures of *E. coli* DH5α, pTA49 and *E. coli* DH5α, pTA50 were grown overnight with Ap and glu. These cultures were then incubated in 25 mL of LB, Ap, and glu in 250-mL flasks at 37°C and 300 rpm from a starting $OD_{600} \approx 0.04$ until the cultures were in the exponential phase ($\approx 1 \times 10^8$ cfu mL^{-1}). Then the bacteria were resuspended in LB, Ap, and L-ara and incubated as described earlier. At specified times, the OD_{600} was measured and samples were removed, washed with PBS, and plated for viable counts at 37°C on LBA, Ap, and glu.

ToxIN Bacteriostatic, Protection, and Stability Assays. Bacteriostatic assays were performed using *E. coli* DH5α, pTA49, pTA76 exactly as for the aforementioned toxicity assays, except that cfu were determined at different times at 37°C on (*i*) LBA, Ap, Sp, and glu and (*ii*) LBA, Ap, Sp, glu, and IPTG. ToxIN protection assays were performed as described for the toxicity assays with the following modifications: A range of different ToxI expression plasmids was used with appropriate antibiotic selection (see *Results* for details), the L-ara induction step was omitted, and the cells were enumerated on LBA, Ap, and Sp plates supplemented with (*i*) glu, (*ii*) glu and IPTG, (*iii*) L-ara, and (*iv*) L-ara and IPTG. ToxIN stability assays using *E. coli* DH5α, pTA49, pTA76 were carried out similarly to the ToxIN protection assays but with cultures grown in LB, Ap, Sp, L-ara, and IPTG (instead of in LB, Ap and glu) before plating for viable counts.

Mapping the Transcriptional Start of *toxIN*. RNA was extracted from *Eca* 1043, pTA46, and 5′ RACE of *toxIN* was performed using the Roche 5′/3′ second-generation RACE kit. cDNA was synthesized using random hexamers and SuperScript II RT (Invitrogen) and the specific primers used were PF146 and PF147. The transcriptional start site of *toxIN* was confirmed by sequencing five 5′ RACE clones. As a further confirmation, RT-PCR was used to validate the location of the 5′ end of the transcript (data not shown).

***toxIN* Promoter *lacZ* Fusion Experiments.** The *toxIN* promoter was amplified using primers PF186 and PF187. To test for a separate *toxN* promoter, the *toxI* region was amplified using primers PF188 and PF189. To examine transcriptional read-through into the toxin gene, the *toxIN* promoter and *toxI* were amplified by PCR using primers PF186 and either PF189 or PF202. The resulting PCR products were cloned into the EcoRI and HindIII sites of pRW50 (39), giving plasmids pTA104, pTA105, pTA106, and pTA119. Promoter expression was determined as described previously (39).

Construction of ToxI Expression Vectors. For ToxI expression vectors compatible with pTA49, a Sm/Sp-resistant derivative of pQE-80L (QIAGEN) was created as follows. First, the Sm/Sp-resistance cassette from miniTn*5*Sm/Sp in strain LIS (39) was cloned by PCR into pQE-80L BspHI sites, using primers PF172 and PF173, resulting in plasmid pTA100. Then a series of *toxI* IPTG-inducible expression vectors with varying numbers of DNA sequence repeats were created, as described below. The *toxI* gene was cloned by PCR into pTA100 EcoRI and HindIII sites using primers PF164 and MJ12. Plasmids were isolated with 5.5 (pTA76), 4.5 (pTA78), 3.5 (pTA79), 2.5 (pTA80), and 1.5 (pTA81) repeats. A 0.5-repeat construct (pTA93) was constructed in the same manner but with PF183 instead of PF164. To generate a plasmid with a single 36-nt repeat unit transcribed precisely from the +1 in the IPTG-inducible promoter in pTA100, PCR was performed with PF185 and PF184 using pQE-80L as the template. The product was digested with XhoI and HindIII and ligated into pTA100 cut with the same enzymes, giving plasmid pTA103. Plasmids that expressed a single mutant repeat containing either silent mutations relative to the predicted peptide coding sequence (pTA107) or a nonsense mutation (pTA122) were created in the same manner as pTA103 but with primers PF190 or PF260, respectively, instead of PF184.

Western Blot Analysis for ToxN. A C-terminally FLAG-tagged *toxN* gene was cloned by PCR into pBAD30 EcoRI and HindIII sites using primers PF137 and MJ13, resulting in pTRB1. For protein samples, cultures of *E. coli* DH5α, pTA76, pTRB1 and *E. coli* DH5α, pTA76, pTA49 were grown as described for the protection assays until reaching an $OD_{600} \approx 0.5–0.8$. Then cultures of each strain were resuspended into 25 mL of LB, Ap, and Sp supplemented with (*i*) glu, (*ii*) glu and IPTG, (*iii*) L-ara, or (*iv*) L-ara and IPTG and grown at 15°C and 300 rpm for 18 h. Western blot analysis was performed against samples from the resulting cultures (normalized to OD_{600}), using a primary rabbit polyclonal anti-FLAG antibody (Sigma–Aldrich) and a goat anti-rabbit HRP conjugated secondary antibody (Sigma–Aldrich) as directed by the manufacturer. Mass spectrometry of ToxN was performed at PNAC, University of Cambridge.

Construction of *Bacillus thuringiensis toxIN* Plasmids. The *B. thuringiensis* $toxI_{Bt}$ and $toxN_{Bt}$ genes were cloned by PCR from plasmid pAW63 into EcoRI and HindIII sites of pTA100 and pBAD30, respectively, using primer pairs PF194–PF196 and PF197–PF195. The resulting plasmids were pTA115 ($toxI_{Bt}$) and pTA117 ($toxN_{Bt}$).

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